

AMENDMENTS TO THE SPECIFICATION

Please amend the paragraph beginning on page 1, line 11 as follows:

The present invention relates to alkaline proteases having high specific activity and strong oxidant resistance, ~~and as an enzyme to~~ As the alkaline proteases of the present invention have an excellent detergency, these enzymes may be added to a detergent, having excellent detergency.

Please amend the paragraph beginning on page 1, line 11 as follows:

Proteases have conventionally been used in a variety of fields such as various detergents (including laundry detergents), cosmetics, bath agents, food modifiers, and pharmaceuticals (such as digestion aids and anti-inflammatory agents). Among them, Of these uses, proteases for detergents are industrially produced in the largest amount and have a great market scale value. A Accordingly, a number of proteases are now put available on the market.

Please amend the paragraph beginning on page 1, line 19 as follows:

In most cases, stains on clothes contain not only proteins but also plural components such as lipids and solid particles. There is accordingly Therefore, there is a demand for detergents having a sufficient detergency high enough to remove such actual complex stains. Finding, from such a viewpoint, To address this demand, the present inventors applied for a patent (WO99/18218), which provided alkaline proteases having a molecular weight of about 43,000 that are capable of retaining caseinolytic activity even in the presence of a high concentration of fatty acids. The alkaline protease provided in WO99/18218 also exhibited and exhibiting excellent detergency even if when the stain is composed of not a simple protein component but plural components, for example, protein and lipid, and having a

~~molecular weight of about 43,000, the present inventors applied a patent (WO99/18218) on them.~~

Please amend the paragraph beginning on page 2, line 8 as follows:

Alkaline proteases ~~superior to the above-described ones in having improved~~ specific activity, oxidant resistance and detergency ~~and that are~~ usable for detergents of wide-ranging compositions ~~have however been requested remain in demand.~~

Please amend the paragraph beginning on page 2, line 14 as follows:

The present inventors searched for ~~such improved~~ alkaline proteases ~~to address the aforementioned demand~~ mainly from enzyme variants. The above-described alkaline proteases ~~are however utterly different~~ possess significant differences in enzymological properties from serine proteases typified by subtilisin, ~~so that Accordingly,~~ the modified site of subtilisin did not provide ~~them with~~ useful information. As a result of a further investigation, the present inventors have found that in order to obtain novel alkaline proteases having improved specific activity, stability against an oxidant and detergency while maintaining the properties of the above-described alkaline proteases, ~~they must have a specific amino acid residue residues must be present at a predetermined position of their amino acid sequence.~~

Please amend the paragraph beginning on page 3, line 4 as follows:

In one aspect of the present invention, ~~there is thus provided is~~ an alkaline protease wherein an amino acid residue at (a) position 84, (b) position 104, (c) position 256 or (d) position 369 of SEQ ID NO:1, or at a position corresponding thereto, has been deleted or ~~selected from: specifically mutated.~~

In the case of position 84 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (a): an arginine residue, residue.

In the case of position 104 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (b): a proline residue.

In the case of position 256 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (e): an alanine, serine, glutamine, valine, leucine, asparagine, glutamic acid or aspartic acid residue, and residue.

In the case of position 369 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (d): an aspartic acid asparagine residue.

Please amend the paragraph beginning on page 3, line 15 as follows:

In another aspect of the present invention, ~~there is also provided is~~ an alkaline protease wherein an amino acid residue at (e) position 66 or 264, (f) position 57, each of 101 to 106, 136, 193 or 342, (g) position 46 or 205, (h) position 54, 119, 138, 148 or 195, (i) position 247, (j) position 124, (k) position 107 or (l) position 257 of SEQ ID NO:1, or at a position corresponding thereto, has been deleted or ~~selected from: specifically mutated.~~

In the case of position 66 or 264 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (e): a glutamine, aspartic acid, serine, glutamic acid, alanine, threonine, leucine, methionine, cysteine, valine, glycine or isoleucine residue, residue.

In the case of position 57, 101 to 106, 136, 193, or 342 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (f): a lysine, serine, glutamine, phenylalanine, valine, arginine, tyrosine, leucine, isoleucine, threonine, methionine, cysteine, tryptophan, aspartic acid, glutamic acid, histidine, proline or alanine residue, residue.

In the case of position 46 or 205 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (g): a tyrosine, tryptophan, alanine, asparagine, glutamic acid, threonine, valine, leucine, isoleucine, histidine, serine, lysine, glutamine, methionine or cysteine residue, residue.

In the case of position 54, 119, 138, 148, or 195 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (h): a tryptophan, phenylalanine, alanine, asparagine, glutamic acid, threonine, valine, histidine, serine, lysine, glutamine, methionine, glycine, aspartic acid, proline, arginine or cysteine residue, residue.

In the case of position 247 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (i): a tryptophan, phenylalanine, alanine, asparagine, glutamic acid, threonine, valine, leucine, isoleucine, histidine, serine, glutamine, methionine or cysteine residue, residue.

In the case of position 124 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (j): an alanine or lysine residue, residue.

In the case of position 107 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (k): a lysine, arginine, alanine or serine residue, and residue.

In the case of position 257 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (l): a valine or isoleucine residue.

Please amend the paragraph beginning on page 5, line 1 as follows:

In a further aspect of the present invention, ~~there are also provided is~~ a gene encoding the alkaline protease, a recombinant vector containing the gene and a transformant containing the vector.

Please amend the paragraph beginning on page 5, line 5 as follows:

In a still further aspect of the present invention, ~~there is also provided is~~ a detergent composition containing the alkaline protease of the present invention.

Please amend the paragraph beginning on page 5, line 10 as follows:

FIG. 1 is a diagram illustrating detergency of an alkaline protease variant. In each of Fig. 1A-1D detergency is illustrated for a detergent lacking the addition of an alkaline protease and a detergent to which the wild type alkaline protease (KP43) is added. Fig. 1A illustrates the detergency for the L104P alkaline protease mutant. Fig. 1B illustrates the detergency for the K84R alkaline protease mutant. Fig. 1C illustrates the detergency for the M256S and M256A alkaline protease mutants. Fig. 1D illustrates the detergency for the D369N alkaline protease mutant.

FIG. 2 is a diagram illustrating relative specific activity of each of alkaline protease variants; and variant as described in Example 5.

FIG. 3 is a diagram illustrating relative residual activity of each a series of alkaline protease variants, in which position 256 of KP43 has been mutated, after treatment with an oxidant.

Please amend the paragraph beginning on page 5, line 18 as follows:

As described above, ~~in~~ the alkaline proteases of the present ~~invention~~, invention includes a deletion or specific mutation of an amino acid residue at (a) position 84, (b) position 104, (c) position 256 or (d) position 369 of SEQ ID NO:1 or at a position corresponding thereto ~~has been deleted or selected from:~~

In the case of position 84 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (a): an arginine residue, residue.

In the case of position 104 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (b): a proline residue.

In the case of position 256 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (c): an alanine, serine, glutamine, valine, leucine, asparagine, glutamic acid or aspartic acid residue, and residue.

In the case of position 369 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (d): an aspartic acid residue, or asparagine residue.

In addition, as described above, the alkaline proteases of the present invention may include a deletion or specific mutation of an amino acid residue at (e) position 66 or 264, (f) position 57, each of 101 to 106, 136, 193 or 342, (g) position 46 or 205, (h) position 54, 119,

138, 148 or 195, (i) position 247, (j) position 124, (k) position 107 or (l) position 257 of SEQ

ID NO:1 or at a position corresponding thereto ~~has been deleted or selected from:~~

In the case of position 66 or 264 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (e): a glutamine, aspartic acid, serine, glutamic acid, alanine, threonine, leucine, methionine, cysteine, valine, glycine or isoleucine ~~residue, residue.~~

In the case of position 57, 101 to 106, 136, 193, or 342 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (f): a lysine, serine, glutamine, phenylalanine, valine, arginine, tyrosine, leucine, isoleucine, threonine, methionine, cysteine, tryptophan, aspartic acid, glutamic acid, histidine, proline or alanine ~~residue, residue.~~

In the case of position 46 or 205 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (g): a tyrosine, tryptophan, alanine, asparagine, glutamic acid, threonine, valine, leucine, isoleucine, histidine, serine, lysine, glutamine, methionine or cysteine ~~residue, residue.~~

In the case of position 54, 119, 138, 148, or 195 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (h): a tryptophan, phenylalanine, alanine, asparagine, glutamic acid, threonine, valine, histidine, serine, lysine, glutamine, methionine, glycine, aspartic acid, proline, arginine or cysteine ~~residue, residue.~~

In the case of position 247 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (i): a tryptophan, phenylalanine, alanine, asparagine, glutamic acid, threonine,

valine, leucine, isoleucine, histidine, serine, glutamine, methionine or cysteine residue, residue.

In the case of position 124 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (j): an alanine or lysine residue, residue.

In the case of position 107 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (k): a lysine, arginine, alanine or serine residue, and residue.

In the case of position 257 of SEQ ID NO:1, or a position corresponding thereto, the preferred mutation is to replace the original amino acid present in the sequence with at position (l): a valine or isoleucine residue.

Please amend the paragraph beginning on page 7, line 7 as follows:

Specifically, the alkaline proteases according to the present invention mean include alkaline proteases having an amino acid sequence represented by SEQ ID NO:1 wherein the amino acid residue at a position selected from the above-described (a) to (d) and (e) to (l) has been deleted or predetermined, or. In addition, the alkaline proteases according to the present invention include another alkaline protease wherein the amino acid residue at a position corresponding thereto to a position selected from the above-described (a) to (d) and (e) to (l) has been deleted or predetermined. They may be The alkaline proteases according to the present invention may include wild type enzymes, wild type variants or artificial variants.

Please amend the paragraph beginning on page 7, line 16 as follows:

The “another alkaline protease” may be a wild type enzyme or a wild type variant. That having In this context it is preferred that the alkaline protease has oxidant resistance and having a molecular weight, as determined by SDS-PAGE, of $43,000 \pm 2,000$ is preferred, of

which that. More preferred is an alkaline protease having an amino acid sequence showing that has at least 60% homology with the amino acid sequence of SEQ ID NO:1 is more preferred. Particularly preferred is that an alkaline protease having an amino acid sequence showing that has at least 60% homology with the amino acid sequence of SEQ ID NO:1, having has oxidant resistance, works on the functions under alkaline side conditions (pH 8 or greater), is stable with at least 80% residual activity when treated at pH 10 for 10 minutes even at 50°C, is inhibited by diisopropyl fluorophosphate (DFP) and phenylmethane sulfonyl fluoride (PMSF) and has a molecular weight, as determined by SDS-PAGE, of 43,000 ± 2,000.

_____ The term “having oxidant resistance” as used herein means that it the alkaline protease has at least 50% of residual activity (synthetic substrate assay) when treated in a 50 mM hydrogen peroxide solution (containing 5 mM calcium chloride) at pH 10 (a 20 mM Britton-Robinson buffer) at 20°C for 20 minutes.

Please amend Table 1-a on page 14 as follows:

Table 1-a

Position	Proteases						
	TS43 KP43 SEQ ID NO:1	9860 SEQ ID NO:2	E-1 SEQ ID NO:3	Ya SEQ ID NO:4	SD-521 SEQ ID NO:5	A-1 SEQ ID NO:6	A-2 SEQ ID NO:7
(a)	84Lys	84Lys	83Lys	83Lys	83Lys	84Lys	83Lys
(b)	104Leu	104Leu	103Leu	103Leu	103Leu	104Leu	103Leu
(c)	256Met	256Met	255Met	255Met	255Met	256Met	255Met
(d)	369Asp	369Asp	368Asp	368Asp	368Asp	369Asp	368Asp

Please amend Table 1-b on page 14 as follows:

Table 1-b

Position	Proteases						
	TS43 KP43 SEQ ID NO:1	9860 SEQ ID NO:2	E-1 SEQ ID NO:3	Ya SEQ ID NO:4	SD-521 SEQ ID NO:5	A-1 SEQ ID NO:6	A-2 SEQ ID NO:7
(e)	66Asn 264Asn	66Asn 264Asn	66Asn 263Asn	66Asn 263Asn	66Asn 263Asn	66Asn 264Asn	66Asn 263Asn
(f)	57Gly	57Gly	56Gly	56Gly	56Gly	57Gly	56Gly
	101Gly	101Ser	100Ser	100Ser	100Ser	101Asn	100Gly
	102Gly	102Gly	101Gly	101Gly	101Gly	102Gly	101Gly
	103Gly	103Gly	102Gly	102Gly	102Gly	103Gly	102Gly
	105Gly	105Gly	104Gly	104Gly	104Gly	105Gly	104Gly
	106Gly	106Gly	105Gly	105Gly	105Gly	106Gly	105Gly
	136Gly	136Gly	135Gly	135Gly	135Gly	136Gly	135Gly
	193Gly	193Gly	192Gly	192Gly	192Gly	193Gly	192Gly
	342Gly	342Gly	341Gly	341Gly	341Gly	342Gly	341Gly
(g)	46Phe 205Phe	46Phe 205Phe	46Phe 204Phe	46Phe 204Phe	46Phe 204Phe	46Phe 205Phe	46Phe 204Phe
(h)	195Tyr	195Tyr	194Ile	194Ile	194Leu	195Tyr	194Tyr
(i)	247Lys	247Lys	246Lys	246Lys	246Lys	247Lys	246Lys
(j)	124Arg	124Arg	123Arg	123Arg	123Arg	124Arg	123Arg
(k)	107Leu	107Leu	106Leu	106Leu	106Leu	107Leu	106Leu
(l)	257Ala	257Ala	256Ala	256Ala	256Ala	257Ala	256Ala

Please amend the paragraph beginning on page 15, line 1 as follows:

When the alkaline protease of the present invention is a variant, the “protease having an amino acid sequence represented by SEQ ID NO:1” or the above-exemplified “another alkaline protease” serves as an alkaline protease prior to mutation (which may be called “parent alkaline protease”). By introducing a mutation to a desired site of this parent alkaline protease, the alkaline protease of the present invention ~~is available~~ may be obtained. For example, ~~it is available~~ the alkaline protease of the present invention may be obtained by deleting or substituting, with another amino acid residue, the amino acid residue at a position selected from the above-described (a) to (d) and (e) to (l) of the amino acid sequence of SEQ

ID NO:1 of (Protease KP43) or at the corresponding position of the amino acid sequence of another alkaline protease, ~~more.~~ More specifically, the amino acid sequence of another alkaline protease may be an amino acid sequence represented by SEQ ID NOS:2 to 7.

Please amend the paragraph beginning on page 16, line 12 as follows:

~~For~~ The following process may be employed for the production of the protease variant of the present invention by using the resulting mutated gene, usable is, for example, the following process. A DNA encoding the protease variant of the present invention is stably amplified by linking the mutated gene with a DNA vector capable of amplifying ~~it~~ stably the same. ~~or by~~ Alternatively, the DNA encoding the protease variant of the present invention is stably amplified by introducing the mutated gene onto a chromosomal DNA capable of maintaining it stably. Subsequent thereto and then, the gene is introduced into a host permitting stable and efficient expression of the gene, whereby the variant protease is produced. Hosts satisfying the above-described conditions include microorganisms belonging to *Bacillus* sp., *Escherichia coli*, mold, yeast and *Actinomyces*.

Please amend the paragraph beginning on page 17, line 1 as follows:

The alkaline protease of the present invention thus obtained has stable protease activity in an alkaline ~~region~~ environment, is free from the inhibition of caseinolytic activity by higher fatty acids, and has a molecular weight, as determined by SDS-PAGE, of 43,000 ± 2,000. For example, the protease variant available from, as a parent strain, the protease having an amino acid sequence of SEQ ID NO:1 has the below-described physicochemical properties.

Please amend the paragraph beginning on page 17, line 15 as follows:

It is stable within a pH range of 6 to 11 ~~under the treating conditions when treated at~~ 40°C for 30 minutes.

Please amend the paragraph beginning on page 21, line 4 as follows:

When the alkaline protease of the present invention is added to a powdery detergent composition, it is preferred to prepare detergent particles in advance ~~and then mix therein.~~
After preparation of the detergent particles, the alkaline protease granules are mixed therein in accordance with the process as described in Japanese Patent Application Laid-Open No. Sho 62-25790 ~~in order~~ to avoid the contact of workers or end users with the enzyme upon preparation or use of the detergent or to prevent heat-induced deactivation or decomposition of the enzyme.